GENOMIC ANALYSIS OF ANTIBIOTIC RESISTANCE IN MICROBIOTA OF HONEY BEES

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Abstract:

The microbiota of an organism plays an essential role in maintaining the health of its host. Gut microbiota serve many functions such as aiding in host immunity and metabolism. When these communities are perturbed, they can deeply affect the livelihood of the host and increase their mortality. Agents such as antibiotics are prime examples of substances that can disrupt the gut microbial communities and weaken the hosts. This ultimately gives opportunistic pathogens the chance to overcome the natural flora. Honey bees are excellent models for studying the interactions between antibiotics and gut microbiota and can be used to further our understanding on these interactions in humans. In beekeeping and agriculture, the use of antibiotics is excessive. In beehives specifically, antibiotics such as tetracycline are used for larval protection against pathogens such as *Serratia* and Foulbrood. This prolonged exposure to antibiotics suggests the acquisition of antibiotic resistance genes in the microbiota of honey bees. Genomic analysis shows the presence of a family of tetracycline efflux pump genes in two core species, *Gilliamella apicola* and *Snodgrassella alvi*, in the honey bee gut. Further genomic analysis of *Serratia* isolated from local hives show the presence of MFS efflux pumps. Minimum Inhibitory Concentration assays performed on strains showing genetic markers for tetracycline resistance showed significant colony growth in the presence of antibiotics, even at the highest tested antibiotic concentrations. Further studies that determine precise inhibitory antibiotic concentrations, potential similarity between transpogenic elements and linkage between antibiotic resistance genes, and specific interactions between *Serratia* strains and core honey bee microbiota species can elucidate lingering questions regarding antibiotics and their effects on the microbiota.

Background:

The microbiota of an organism is important to the organism's health and quality of life. Functions of the microbiota in humans consist of nutrient metabolism, drug metabolism, and immunity against pathogens [Jandhyala et al., 2015]. Recent studies have shown that the microbiota can also influence the behavior and function of the central nervous system in an organism [Neufeld and Foster, 2009]. Studying the microbiota is essential in understanding the deeper connections microbes have in maintaining the health of different organisms.

The gut microbial populations are highly sensitive and can be disturbed by several things: the most potent cause of these disturbances is antibiotics. Although antibiotics have been beneficial in numerous ways, these substances are known to impact the composition and size of the microbiota [Ng et al., 2013]. Studies have shown that dysbiotic microbiomes have impaired pathogen protection, nutrient metabolism, and vitamin production functions [Langdon et al., 2016]. Upon treatment of antibiotics, the changes in the gut microbiota can be disturbed for weeks and sometimes cannot be restored to the original composition [Langdon et al., 2016]. The disturbed diversity of the microbiota makes host organisms more susceptible to opportunistic pathogens since the host no longer has the natural flora to defend against the pathogens [Langdon et al., 2016]. It is thus extremely important to study the effects antibiotics has on the gut flora of humans to help determine a better way of administrating antibiotics that does not further compromise the health of humans. Furthermore, studying how antibiotics perturbs the gut flora can help create more specific antibiotic treatment plans for sick individuals. Knowing more causative relations between microbiota, health, and antibiotic treatments can help evolve a method of treating bacterial infections more specifically such that it targets only harmful bacteria and not members of the original bacterial communities [Lemon et al, 2012].

Not only do antibiotics have negative effects on our microbiomes, but they also have negative effects on our ecosystem's health. The growing use of antibiotics has led to a stark increase in the development of antibiotic resistant bacteria. The growing number of these antibiotic resistant bacteria pose a significant concern to the maintenance of a healthy gut microbiome. The development of antibiotic resistance in microbes comes about from either rare mutational events in bacterial genomes or from acquisition of resistant genes found in the environment [Cabello et al., 2006]. Acquiring these genes allows bacteria to respond differently in the presence of antibiotics, either structurally or metabolically [Witte, 1998].

The sharp increase in antibiotic resistance in microbial communities stems from the over usage of antibiotics in agriculture, where antibiotic usage is often unregulated and administered in copious amounts. For example, studies in Denmark have showed that 24 grams of glycopeptide vancomycin were used in human therapy, whereas 24,000 kilograms of a similar glycopeptide avaparcin were used in animal feed to enhance and protect livestock [Witte,1998]. It is undeniable that human and animal microbes are connected, and the copious amounts of antibiotics given to animals will affect resistance levels in animals and subsequently humans as well.

Honeybee populations across the country have had long exposure to antibiotics for combating bacterial diseases such as foulbrood that affects larvae of honeybees. Over the span of 50 years, bee breeders across the United States have been using tetracycline to treat for these pathogens [Tian et al., 2012]. The prolonged exposure to these antibiotics had led to the increase of resistance in these communities, especially among pathogenic bacteria such as *Paenibacillus larvae* and *Serratia marcescens* which affect honeybee larvae [Climaco, 2017]. The combination of antibiotics causing dysbiosis in gut flora with the growing resistance of these pathogenic

bacteria have contributed to high mortality rates observed in honeybee populations [Raymann et al., 2017].

Honey bees, or *Apis mellifera*, are good model organisms that can be utilized to study the microbiota functions in humans. In humans, the microbiome fingerprint of each individual has complex interactions with the body that play a part in maintaining health. So far these results are purely correlative as there is not sufficient information to suggest causative relations between changes in microbiota and health. Honey bees are prime organisms in studying these interactions as they too have unique microbiome fingerprints similar to humans. Furthermore, bees acquire their microbiota through communal contact rather than external sources as do humans [Powell et al., 2014]. Furthermore, honey bees have much simpler digestive systems and are thus easier to manipulate. As a result, it is easier to gather causative data between changes in microbiota and changes in physiology in the honey bee. Trends determined in the honey bee can be used to further our understanding of the human gut microbiota.

This study specifically focusses on *Gilliamella apicola* and *Snodgrassella alvi,* two known symbionts found in the ileum of honeybees [Kwong et al., 2014]. Their shared location in the honeybee guts presents an ideal opportunity to exchange antibiotic resistance genes through horizontal gene transfer. In this study, genomes of 53 isolates *G. apicola* and 32 isolates of *S. alvi* were scanned for of the presence of 17 tetracycline resistance genes to determine the prevalence and distribution of resistance genes. These 17 genes originate from two families of tetracycline resistant genes: one family consists of genes that encode for tetracycline efflux pumps and the other consists of genes that encode for ribosomal protection proteins. Species of *S. marcescens* isolated from lab beehives were also investigated to determine which tetracycline genes were prevalent. Minimum inhibitory concentration tests were conducted on bacterial

strains with tetracycline resistance genes to determine the level of resistance. These studies could illuminate how antibiotics like tetracycline are affecting honeybee microbiomes and help further our understanding of antibiotic effects on gut bacteria in honeybees and by extension, humans.

Methods:

Media for Bacteria 4

In this study, *Gilliamella apicola*, *Snodgrassella alvi*, and a variety of pathogenic *Serratia* isolates were studied. Different growth media were utilized for optimal growth to cultivate these bacteria from freezer stocks. *G. apicola* and *S. alvi* were grown on blood agar plates comprising of Heart Infusion Agar from Criterion. Following provided instructions, agar was made and sterilized in the autoclave. Once cooled to 33°C, sheep blood was added to the media in a 5% blood to agar ratio. *G. apicola* and *S. alvi* grown on these plates were incubated in a 35°C CO_2 incubator. *Serratia* species were grown on LB agar plates. These plates comprised of NaCL from Fisher Scientific, Bacto Tryptone from Becton Dickinson and Co., Yeast Extract from Affymetrix, and agar from Amresco. Bacteria colonized on these plates were incubated in a 35°C CO_2 incubator.

Minimum Inhibitory Concentration Plate experiment

Minimum Inhibitory Concentration experiments are used to identify the minimal concentrations of antibacterial compounds necessary to inhibit microbial growth. Using a stock tetracycline solution of 12.5 mg/ml, agar media with tetracycline concentrations of 4 µg/ml, 8 µg/ml, 16 µg/ml, 32 µg/ml were made. Blood agar media was made for *G. apicola* and *S. alvi* tests with the same protocol described above. Tetracycline was added with the sheep blood to create a homogenous mixture. The same method was used to make LB media with tetracycline for the *Serratia* MIC assays.

Freezer stocks of 5 *S. alvi* species, 6 *G. apicola* species, and 4 *Serratia* species with known genetic markers for tetracycline resistance were plated on their ideal growth media in a 35°C CO_2 incubator for 48-72 hours. Following incubation, 3 to 5 medium sized colonies were

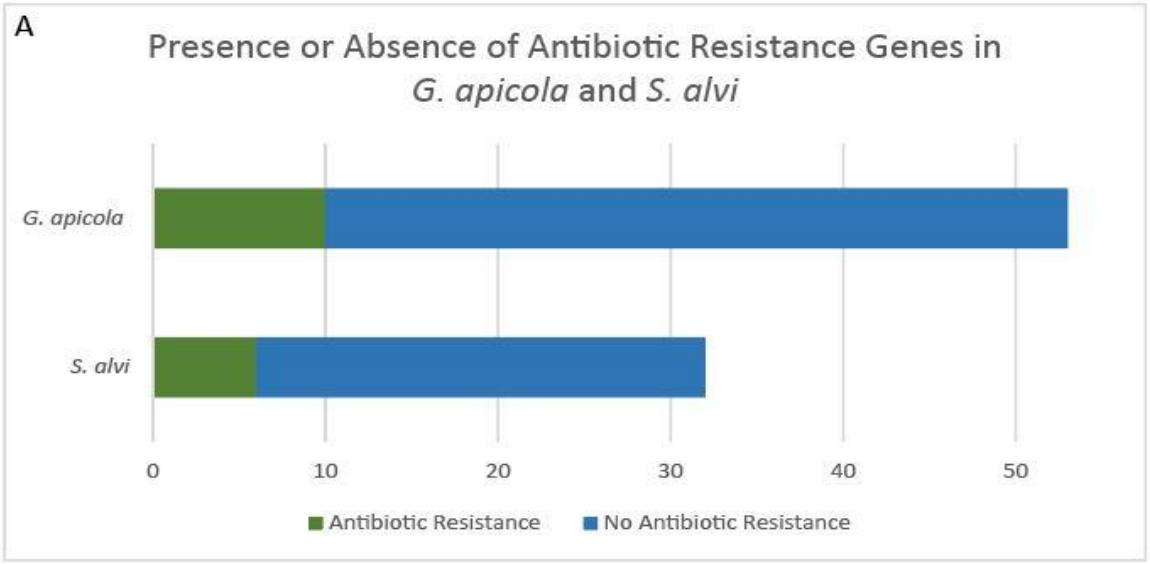
selected from plates and inoculated in 1 ml of 1X Phosphate-buffered saline buffer. Large inoculation loops were rotated in micro-centrifuge tubes to break down large colonies. Solutions were briefly vortexed for 5 seconds. Each of the bacterial solutions were serially diluted to a 10^{-4} cell per milliliter concentration. Fifty microliters of the 10^{-4} solution were pipetted on the appropriate tetracycline media plates. Three millimeter sterile glass beads were used to equally distribute solution across the agar. The plates remained at room temperature to dry and were then incubated in a 35°C CO_2 incubator. *Serratia* plates were incubated for 48 hours before colonies were counted and *S. alvi* and *G. apicola* plates were checked between 48-72 hours depending on bacterial growth.

BLAST Database

BLAST+ software was used to create databases of *G. apicola*, *S. alvi*, and *Serratia* genomes [BLAST+, 2004]. Genomes of these bacteria were compiled from different research projects and concatted and formatted into individual databases. Seven tetracycline resistance gene sequences that encode for ribosomal protection proteins against the antibacterial were retrieved from different species in a phylogenetic study conducted by Aminov et al. [Aminov et al., 2001]. Ten tetracycline resistance gene sequences utilizing efflux channels to remove tetracycline were retrieved from a variety of species in a different phylogenetic study conducted by Aminov et al. [Aminov et al., 2002]. These individual genes were located and concatenated into a query database and were compared against the genomic databases of these three bacterial species from bee guts. Bacterial species, percent identity, percent difference, bit scores, and e-values were documented for each match between tetracycline genes and bacterial genomes. Code utilized in the experiment is provided in Appendix A.

Results:

This study focusses on two bacterial symbionts that reside in the ileum of the honey bee gut. Complete genomes of 53 *Gilliamella apicola* and 32 *Snodgrassella alvi* strains were assembled and concatenated in two separate BLAST databases. Of the tested *G. apicola*, 53% of the strains were isolated from different bumble bee hosts and 28% of the strains were cultivated from honey bee hosts. Of the tested *S. alvi* strains, 62.5% of strains were cultivated from bumble bee hosts and 31% of strains were cultivated from honey bee hosts.



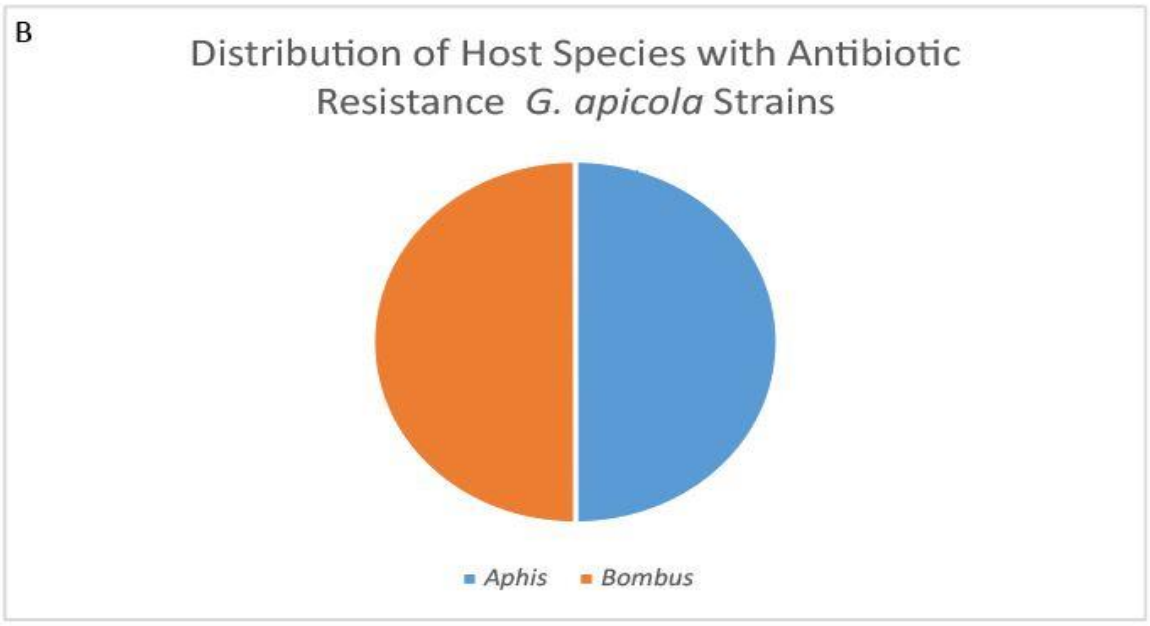


Figure 1. Presence of Tetracycline resistance in bee gut symbionts *(A.)Presence or absence of Tetracycline resistance genes in S. alvi and G. apicola in bee gut microbiota. Manual BLASTn databases were made to assay 17 Tetracycline genes against 53 G. apicola and 32 S. alvi genomes. Bar lengths indicate proportion of antibiotic resistance strains to nonresistant strains from both families of bacteria. (B.) Distribution of hosts of tested G. apicola strains that showed presence of antibiotic resistance.*

Bumble bee and honey bee species were cultivated from different locations around the United States and parts of the world to get a wide distribution of test subjects. Seventeen tetracycline resistant genes from two different families of genes were collected from studies done by Aminov et al. in 2001 and 2002. BLAST+ was used to create databases and assay for isolated tetracycline resistance genes.

Of the tested genomes, 10 out of 53 of *G. apicola* strains, or 18.8%, had antibiotic resistance genes (Figure 1A). Out of the 10 strains with evidence of antibiotic resistance genes, 2 strains had more than one antibiotic resistance genes present (Figure 2). Of the tested *S. alvi* strains, 6 out of the 32 strains, or 18.75%, of the strains had antibiotic resistance genes (Figure 1A). All of the *S. alvi* strains with antibiotic resistance came from *Apis mellifera* species. Of the 10 strains of *G. apicola* with tetracycline resistance, 5 of these strains came from different *Bombus* hosts and 5 came from different *Apis* hosts (Figure 1B).

Two families of tetracycline resistance genes were tested in these experiments. One family, tetracycline genes A through J, are phylogenetically related genes that encode for efflux pumps on cell membranes that evacuate molecules of tetracycline from the cell. The other family of genes, tetracycline genes M through Z, are also phylogenetically related and are genes that encode for ribosomal protection proteins. In all of the strains of *S. alvi* and *G. apicola* all

antibiotic resistance genes encoded ~~for~~ efflux pumps (Figure 2). None of the *Serratia, S. alvi,* and *G. apicola* strains had tetracycline resistance genes that encoded ribosomal protection proteins (Figure 2).

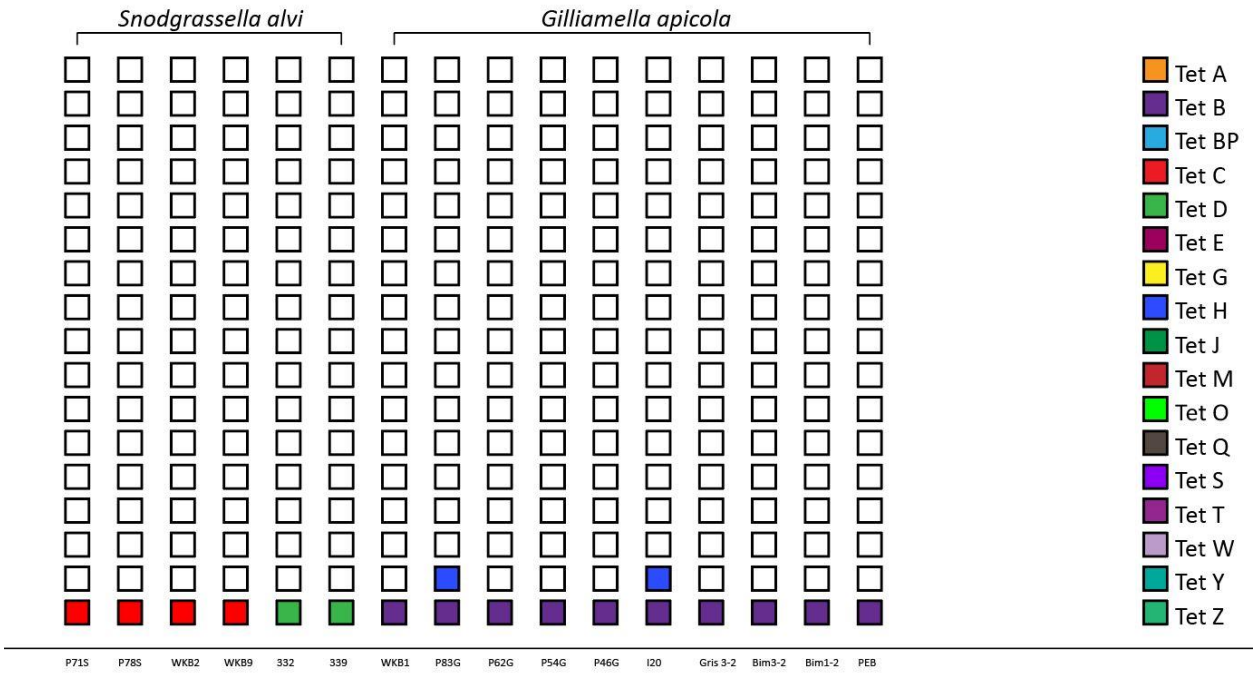


Figure 2. Distribution of Tetracycline resistance genes in resistant bee gut microbiota

Occurrence of 4 tetracycline genes in resistance strains of G. apicola and S. alvi (13 other genes were screened but not found). Different colors indicate presence of a particular gene which white boxes indicate no matches. Detailed information regarding each of the isolated tetracycline genes is found in Appendix B. Figure adapted from Tian et al. 2012 paper.

After assaying two natural resident symbiotic bacteria in bee gut microbiota, known honey bee pathogens were tested for antibiotic resistance. Genomes of 4 *Serratia* strains were sequenced, and tested for the presence of 17 tetracycline resistance genes. Genome sequences of *Serratia* strains KZ2, SS1, KZ11, and KZ19 were acquired from Raymann et al. Genomes of the four *Serratia* strains were concatenated in a locally created BLAST database and assayed for the

presence of the 17 tetracycline resistance genes. The results show no indication of any of these genes being present in the *Serratia* strains. However, genomes of all four *Serratia* showed annotations for tetracycline resistance genes. Examination of these genes showed the presence of tetracycline resistance MFS efflux pumps that are predominately found in Gram positive bacteria [Sun et al., 2014]. This MFS efflux pump gene was found in all four of the tested *Serratia* strains.

A.	**KZ11 (MFS)**	**KZ19 (MFS)**	**SS1 (MFS)**	**KZ2 (MFS)**
4 µg/ml	294	13	29	4
8 µg/ml	218	20	39	6
16 µg/ml	151	6	28	5
32 µg/ml	69	0	1	4

B.	**WKB7 (no tet gene)**	**WKB112 (no tet gene)**	**P62G (tetB)**	**WKB1 (tetB)**	**P83G (tetB and tetH)**	**P54G (tetB)**
4 µg/ml	0	0	580	281	30	TMTC
8 µg/ml	0	0	560	368	32	TMTC
16 µg/ml	0	0	278	TMTC	28	TMTC
32 µg/ml	0	0	46	13	5	92

C.	**WKB273 (no tet gene)**	**WKB2 (tetC)**	**339 (tetD)**	**332 (tetD)**	**WKB9 (tetC)**
4 µg/ml	0	529	75	TMTC	19
8 µg/ml	0	457	40	27	17
16 µg/ml	0	TMTC	TMTC	23	14
32 µg/ml	0	318	53	17	14

Table 1. Colony counts of Minimum Inhibitory Concentration Assays for *G. apicola, S. alvi,* and *Serratia* strains *Colony growth of different antibiotic resistant and nonresistant bacteria strains of Serratia (A), G. apicola (B), and S. alvi (C) on media with varied concentrations of Tetracycline. Plates with confluent or dense growth were labeled as TMTC, or too many to count.*

Minimum Inhibitory Concentration Assays were conducted next to determine concentrations of tetracycline that inhibited growth of *Serratia*, *G. apicola*, and *S. alvi*. Four different concentrations of tetracycline were tested against 4 strains of *Serratia*, 6 strains of *G. apicola*, and 5 strains of *S. alvi*. All strains of *S. alvi* and *G. apicola* with and without antibiotic resistance genes selected for the study originated from different *Apis* hosts. Of the *G. apicola* strains tested in these assays, strains identified as WKB7 and WKB112 were tested as controls since they showed no evidence of tetracycline resistance genes in their genomes. Of the *S. alvi* strains tested, the strain identified as WKB273 did not have evidence of tetracycline resistance genes in its genomes and served as a control in this experiments. All of the tested strains in the MIC assays showed confluent growth on media with no tetracycline present. Thus, for the purposes of this experiment, colony counts in environments with no tetracycline was excluded.

In the tested *S. alvi* strains, all strains showed growth on the 32 µg/ml plates, suggesting high resistance to tetracycline. For strains WKB2 and WKB9, the highest growth is observed on the lowest tetracycline concentrations and the lowest growth is observed on the highest tetracycline concentrations. Both strains showed substantial growth on the highest tetracycline concentrations suggesting that 32 µg/ml of tetracycline was not enough to completely subdue growth of both bacterial strains. WKB2 showed increased growth at 16 µg/ml tetracycline and then markedly less growth at a 32 µg/ml of tetracycline. It is unclear as to why there was an increase in growth at that concentration. However, growth patterns of WKB2 and WKB9 strains show that TetC gene is able to sustain growth for these bacteria at high concentrations of tetracycline (Figure 3). *S. alvi* strains 339 and 332 showed similar trends as the previous two bacteria, displaying high growth at low tetracycline concentrations and low growth in higher concentrations. Strain 339's growth pattern showed an unexpected dip at 8 µg/ml of tetracycline,

peak at 16 µg/ml of tetracycline, then dip again at the highest tetracycline concentration. It is unclear as to why there was dense growth at 16 µg/ml of tetracycline and not at lower concentrations of tetracycline. In all, strains 339 and 332 growth patterns suggest that TetD is also able to sustain bacterial growth in the presence of high concentrations of tetracycline (Figure 3).

Of the six strains of *G. apicola* tested, 4 had tetracycline resistance genes. Strain P62G showed a predictable reduction of growth from the low concentrations of tetracycline to high concentrations. At 32 µg/ml of tetracycline there were still 46 colonies observed which indicates that this concentration was not high enough to eliminate bacterial growth completely (Table 1B). Further study can be done with higher concentrations of tetracycline to determine the concentration of antibiotic that eliminates growth. The growth pattern of WKB1 showed some anomalies, as number of colonies observed at 8 µg/ml and 16 µg/ml were higher than the lowest and highest concentrations of tetracycline. Strain P83G showed decreased growth as tetracycline concentrations increased. Strain P54G showed decreased bacterial growth between 16 µg/ml and 32 µg/ml of tetracycline suggesting that slightly higher concentrations of tetracycline would be sufficient to inhibit growth of P54G.

Serratia strain KZ19 showed no growth at 32 µg/ml of tetracycline indicating that a concentration between 16 µg/ml and 32 µg/ml or 32 µg/ml of tetracycline was sufficient to inhibit growth (Table 1A). Further study can be done at small increments to determine the exact concentration at which growth is inhibited. *Serratia* strain SS1 showed high amounts of growth at lower tetracycline concentrations and at 32 µg/ml of tetracycline, only 1 colony was observed (Table 1A). It can be concluded that tetracycline concentrations slightly higher than 32 µg/ml would be sufficient to inhibit growth of strain SS1. Strain KZ2 showed relatively steady growth

between all concentrations of tetracycline tested (Table 1A). This indicates that growth is not greatly affected as tetracycline concentrations in the environment increases. Strain KZ11 showed significant growth in the tested tetracycline concentrations (Table 1A). There were still 69 colonies observed at 32 µg/ml concentration of tetracycline suggesting that KZ11 colonies were still able to sustain growth at that concentration (Table 1A). Further study needs to be done in order to determine concentration that inhibits growth.

Discussion and Future Study:

Honey bee microbiota is comprised of a specific gut community whose interactions promote the health of the honey bees. Through the use of agents like antibiotics, these interactions can be weakened or lost and can subsequently impact host health. Understanding the interactions between the microbiota and antibiotics can aid in our understanding of these interactions in humans and other animals.

The prominent use of antibiotics in the agricultural industry allows for the proliferation of antibiotics in our natural environment. The introduction of antibiotics in the natural environment stems from the large increase in livestock in the agricultural industry. [Khachatouriansm, 1998] With the purpose of antibiotics promoting growth in livestock, the increased amount of livestock in the industry leads to this increased use of antibiotics. [Khachatouriansm, 1998] The livestock in agriculture use 100 to 1000 times more antibiotics that humans each year. [Feinmen, 1998] A longitudinal study in Wisconsin showed that the increase of 0.25 kg to 1 kg per ton of antibiotics such as oxytetracycline being administered to livestock correlated with increased antibiotic resistance in certain *E. coli* species. [Khachatourians, 1998] The sheer amount of antibiotics in the environment and its prolonged usage contributes to the increase of antibiotic resistance in microbial communities not only within livestock, but also in other organisms like honey bees. The strains of *G. apicola* and *S. alvi* tested in the study originated from hives that supposedly were not exposed to antibiotics; however, there is some evidence of antibiotic resistance within the microbiota. This shows that antibiotics present in the environment is enough for microbes in different communities to develop antibiotic resistance, even if they aren't exposed directly.

Tetracycline is a broad spectrum antibiotic that has been used in bee hives for decades [Tian et al., 2012]. Due to its prolonged use in bee keeping, the possibility of microbes in bee

communities developing resistance to these antibiotics increases. Genomic analysis of isolated *G. apicola* and *S. alvi* from different honey bee and bumble bee species show that tetracycline resistance genes, specifically TetB, TetH, TetC, and TetD, were found. These genes share ancestry, and all encode for tetracycline efflux pumps. These results show that the prolonged exposure of antibiotics in honeybees has allowed for the development of antibiotic resistance. However, of the 85 microbial genomes tested, only 16 genomes showed genetic markers of antibiotic resistance. This suggests that antibiotic resistance is present in these communities but at very low levels. Furthermore, the combination of results from the genetic analyses and MIC assays show that the presence of tetracycline resistance genes in the genomes are good indicators of the ability for these bacteria to withstand the effects of tetracycline. This shows that the ability to tolerate tetracycline is derived from the presence of the assayed genes and not from some unknown resistance genes with unrecognizable genetic sequences.

The consequences of antibiotic treatments can be seen in their effects on natural gut flora. A study by Raymann et al. show that antibiotic treatment affects the microbial makeup of the honey bee gut, making the gut more susceptible to opportunistic pathogens like *Serratia* or American Foulbroud [Raymann et al., 2017]. These findings are substantiated by genomic analysis of *G. apicola* and *S. alvi* strains in this study. Due to their low level of observed resistance, it is expected that when treated with tetracycline, many of the bacterial strains will not be able to sustain growth. Moreover, the Raymann et al. study showed that the relative abundance of *G. apicola* increased a few days post treatment of tetracycline [Raymann et al., 2017]. The high abundance of tetracycline resistance genes in *G. apicola* provides an explanation as to why this core gut species was able to withstand and grow under the presence of antibiotics. Colony counts from the MIC experiments further show that *G. apicola* strains with antibiotic

resistance genes are able to demonstrate significant growth in the presence of antibiotics. One particular *G. apicola* strain, P83G, from an *Apis mellifera* host had both Tet H and Tet B resistance genes in its genome. If this strain is found in many hosts, it might enhance host survival when exposed to antibiotics. Further study could determine if the *G. apicola* strains that showed increased abundance in the Raymann et al. study had these genes in their genomes. Since strains of *G. apicola* with resistance genes can withstand high concentrations of tetracycline, as MIC assay results show, it is possible that these resistant *G. apicola* strains can be fed to honey bees to allow for *G. apicola* recolonization post treatment of tetracycline. Further study can be done to see if this would be enough to restore *G. apicola* populations in the gut microbiota and combat the emergence of pathogenic bacteria.

G. apicola and *S. alvi* are both Gram negative bacteria that inhabit the ileum of the honey bee gut [Kwong et al., 2014]. These two bacteria have been shown to coevolve in the honey bee gut and can interchange genes through horizontal gene transfer [Kwong et al., 2014]. It was expected that because of these interactions there would be a high similarity in the genes found in both bacteria. The differences in observed genes suggest that these four genes were acquired independently or from other microbes found in the honey bee. Studies have shown that core species of the honey bee gut can carry antibiotic resistance and these results corroborate these findings [Raymann et al., 2017]. TetC, TetD, TetB, and TetH are all from the same family of genes and have similar mechanisms of protecting microbes against antibiotics. The similar family type of gene found between both species indicates a symbiotic relationship between both bacterial species. This indicates a coevolution between the two species which provides an explanation for the development of similar methods to combat exposure to antibiotics. Further study can be done to determine more closely the mechanisms behind the development of

antibiotic resistance genes in these microbes. Co-culturing studies can be done to show if direct transfers of these genes between these two symbionts is possible since the genomes of *G. apicola* and *S. alvi* assayed show no gene similarities. This would further verify that these bacteria can transfer genes amongst themselves in order to enhance gut microbial growth. Furthermore, transpogenic elements allowing these genes to transfer from microbe to microbe can be tested to determine if there is a link between specific transposons and genes. Although tetracycline resistance is primarily focused on in bee populations since tetracycline is a common treatment agent in bee hives, there is a possibility of linked coresistances developing following exposure to single antibiotics. [Levy and Marshal, 2013]. Genomic analysis for the presence of ampicillin resistance genes can also be done to determine whether there is any linkage between tetracycline and ampicillin resistant genes found in these gut microbes. Since antibiotics have the potential of significantly perturbing gut microbiota, determining genetic linkages between transmitted genes can give better insight as to how antibiotic usage effects gut microbes on a genetic level.

Genetic analysis in this study found that *Serratia* strains isolated from different *Apis mellifera* hosts had genes for major facilitator superfamily (MFS) pumps [Kumar et al., 2013]. *Serratia* are opportunistic pathogens that are present in perturbed microbial communities of the honey bee gut [Moran et al., 2012]. The presence of *Serratia* and exposure to tetracycline have showed higher mortality in bees suggesting that *Serratia* can thrive on the dysbiosis in the microbiota caused by the antibiotics [Raymann et al., 2017]. This is supported by the findings of these MFS efflux genes found in *Serratia* that allow these strains to survive exposure to antibiotics. Minimum inhibitory concentration experiments show that most of the *Serratia* are able to maintain growth in high tetracycline concentrations such as 32 µl/mg. The ability for

Serratia to withstand these high concentrations would allow it to take advantage of the dysbiosis from antibiotic treatment.

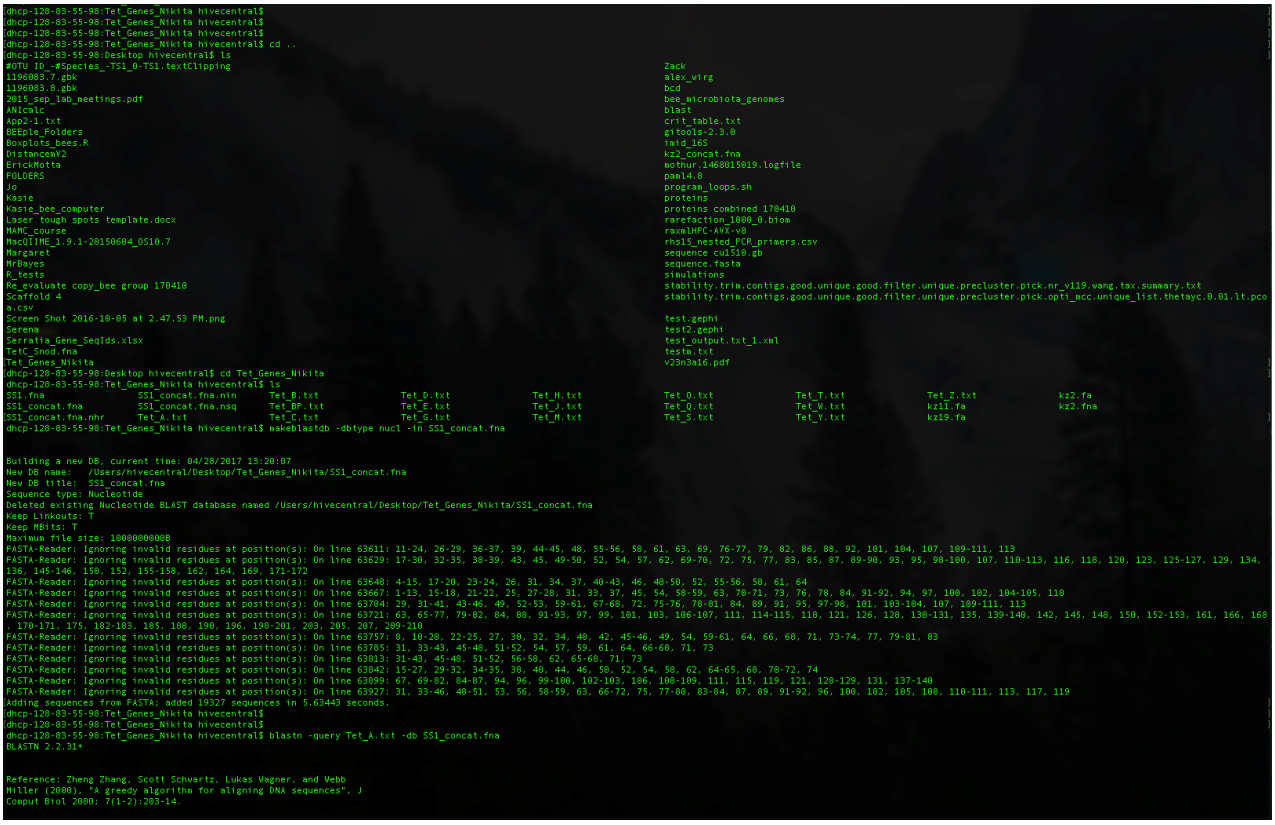
Maintaining a healthy composition of microbes in the gut flora contributes greatly to the overall health of the organism and can aid in nutrition, immunity, and metabolism in hosts [Johnson et al., 2016]. Disturbance, whether an abundance in species or lack of, in these communities can lead to deficiencies in these functions and can ultimately result in death of the host. The presence of certain antibiotic resistance genes in *G. apicola* could explain the increased persistence in the gut with the introduction of tetracycline. When tested to see if growth is sustained at 32 µl/mg of tetracycline, high colony counts were observed for *G. apicola* and *S. alvi* strains with antibiotic resistance. What is interesting is that the *G. apicola* strain P83G had two antibiotic resistance genes detected in the genome; however, its ability to grow at high tetracycline concentrations was markedly less than other *G. apicola* strains that had one detected gene. Less growth could have been a result of both of the genes not being activated. Further studies should be done to determine whether both genes were expressed or whether certain circumstances activate expression of one gene over the other.

Results from this study give some more insight into the realm of antibiotic resistance but there is further study that can be conducted. Further genomic study can be done with the tetracycline gene markers present in the genomes. Since *G. apicola* and *S. alvi* are symbionts in the ileum, horizontal gene transfer can be a method of exchanging genes. Determining a link can further illuminate the why *G. apicola* and *S. alvi* strains did not share the same genes. Further study can also be done between these symbionts and *Serratia* strains in bees. In this study it was observed that *G. apicola* and *S. alvi* strains were able to sustain much more growth than *Serratia* at similar tetracycline concentration. This implies that if these microbes grow in the same

environment, these core gut microbes could potentially overgrow the *Serratia.* Live bee experiments and microbiology experiments can be done to further investigate how tetracycline resistance strains of *Serratia, G. apicola,* and *S. alvi* strains interact to determine what specifically makes bees so susceptible to these pathogens after treatment of antibiotics despite growth of core species in the natural flora.

Overall, findings from this study attempt to address lingering questions about antibiotic resistance in honey bee gut microbes and pathogenic *Serratia.* Further studies and applications of these findings can be used to deepen our understanding of the relationship between gut microbiota and organism health.

Appendix A: Sample code for BLAST database built



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ANIcalc                                                                     blast
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DistanceW2                                                                  kz2_concat.fna
ErickMotta                                                                  mothur.1468015019.logfile
FOLDERS                                                                     paml4.8
Jo                                                                          program_loops.sh
Kasie                                                                       proteins
Kasie_bee_computer                                                          proteins combined 170410
Laser tough spots template.docx                                             rarefaction_1000_0.biom
MAMC_course                                                                 raxmlHPC-AVX-v8
MacQIIME_1.9.1-20150604_OS10.7                                              rhs15_nested_PCR_primers.csv
Margaret                                                                    sequence cu1510.gb
MrBayes                                                                     sequence.fasta
R_tests                                                                     simulations
Re_evaluate copy_bee group 170410                                           stability.trim.contigs.good.unique.good.filter.unique.precluster.pick.nr_v119.wang.tax.summary.txt
Scaffold 4                                                                  stability.trim.contigs.good.unique.good.filter.unique.precluster.pick.opti_mcc.unique_list.thetayc.0.01.lt.pco
a.csv
Screen Shot 2016-10-05 at 2.47.53 PM.png                                    test.gephi
Serena                                                                      test2.gephi
Serratia_Gene_SeqIds.xlsx                                                   test_output.txt_1.xml
TetC_Snod.fna                                                               testm.txt
Tet_Genes_Nikita                                                            v23n3a16.pdf
dhcp-128-83-55-98:Desktop hivecentral$ cd Tet_Genes_Nikita
dhcp-128-83-55-98:Tet_Genes_Nikita hivecentral$ ls
SS1.fna             SS1_concat.fna.nin      Tet_B.txt           Tet_D.txt           Tet_H.txt           Tet_O.txt           Tet_T.txt           Tet_Z.txt           kz2.fa
SS1_concat.fna      SS1_concat.fna.nsq      Tet_BP.txt          Tet_E.txt           Tet_J.txt           Tet_Q.txt           Tet_W.txt           kz11.fa             kz2.fna
SS1_concat.fna.nhr  Tet_A.txt               Tet_C.txt           Tet_G.txt           Tet_M.txt           Tet_S.txt           Tet_Y.txt           kz19.fa
dhcp-128-83-55-98:Tet_Genes_Nikita hivecentral$ makeblastdb -dbtype nucl -in SS1_concat.fna

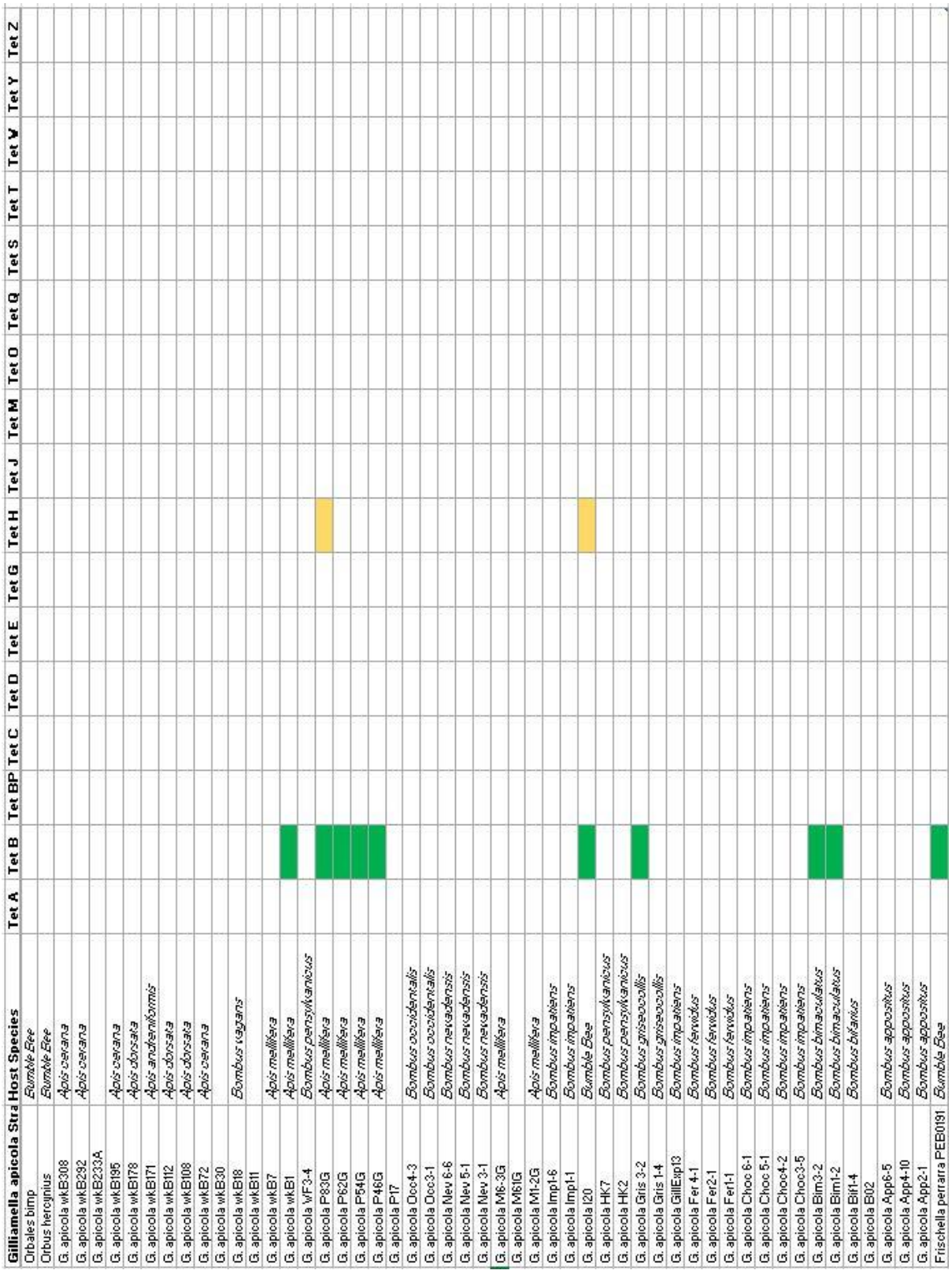

Building a new DB, current time: 04/28/2017 13:20:07
New DB name:   /Users/hivecentral/Desktop/Tet_Genes_Nikita/SS1_concat.fna
New DB title:  SS1_concat.fna
Sequence type: Nucleotide
Deleted existing Nucleotide BLAST database named /Users/hivecentral/Desktop/Tet_Genes_Nikita/SS1_concat.fna
Keep Linkouts: T
Keep MBits: T
Maxbaum file size: 1000000000B
FASTA-Reader: Ignoring invalid residues at position(s): On line 63611: 11-24, 26-29, 36-37, 39, 44-45, 48, 55-56, 58, 61, 63, 69, 76-77, 79, 82, 86, 88, 92, 101, 104, 107, 109-111, 113
FASTA-Reader: Ignoring invalid residues at position(s): On line 63629: 17-30, 32-35, 38-39, 43, 45, 49-50, 52, 54, 57, 62, 69-70, 72, 75, 77, 83, 85, 87, 89-90, 93, 95, 98-100, 107, 110-113, 116, 118, 120, 123, 125-127, 129, 134,
136, 145-146, 150, 152, 155-158, 162, 164, 169, 171-172
FASTA-Reader: Ignoring invalid residues at position(s): On line 63648: 4-15, 17-20, 23-24, 26, 31, 34, 37, 40-43, 46, 48-50, 52, 55-56, 58, 61, 64
FASTA-Reader: Ignoring invalid residues at position(s): On line 63667: 1-13, 15-18, 21-22, 25, 27-28, 31, 33, 37, 45, 54, 58-59, 63, 70-71, 73, 76, 78, 84, 91-92, 94, 97, 100, 102, 104-105, 110
FASTA-Reader: Ignoring invalid residues at position(s): On line 63704: 29, 31-41, 43-46, 49, 52-53, 59-61, 67-68, 72, 75-76, 78-81, 84, 89, 91, 95, 97-98, 101, 103-104, 107, 109-111, 113
FASTA-Reader: Ignoring invalid residues at position(s): On line 63721: 63, 65-77, 79-82, 84, 88, 91-93, 97, 99, 101, 103, 106-107, 111, 114-115, 118, 121, 126, 128, 130-131, 135, 139-140, 142, 145, 148, 150, 152-153, 161, 166, 168
, 170-171, 175, 182-183, 185, 188, 190, 196, 198-201, 203, 205, 207, 209-210
FASTA-Reader: Ignoring invalid residues at position(s): On line 63757: 8, 10-20, 22-25, 27, 30, 32, 34, 40, 42, 45-46, 49, 54, 59-61, 64, 66, 68, 71, 73-74, 77, 79-81, 83
FASTA-Reader: Ignoring invalid residues at position(s): On line 63785: 31, 33-43, 45-48, 51-52, 54, 57, 59, 61, 64, 66-68, 71, 73
FASTA-Reader: Ignoring invalid residues at position(s): On line 63813: 31-43, 45-48, 51-52, 56-58, 62, 65-68, 71, 73
FASTA-Reader: Ignoring invalid residues at position(s): On line 63842: 15-27, 29-32, 34-35, 38, 40, 44, 46, 50, 52, 54, 58, 62, 64-65, 68, 70-72, 74
FASTA-Reader: Ignoring invalid residues at position(s): On line 63899: 67, 69-82, 84-87, 94, 96, 99-100, 102-103, 106, 108-109, 111, 115, 119, 121, 128-129, 131, 137-140
FASTA-Reader: Ignoring invalid residues at position(s): On line 63927: 31, 33-46, 48-51, 53, 56, 58-59, 63, 66-72, 75, 77-80, 83-84, 87, 89, 91-92, 96, 100, 102, 105, 108, 110-111, 113, 117, 119
Adding sequences from FASTA; added 19327 sequences in 5.63443 seconds.
dhcp-128-83-55-98:Tet_Genes_Nikita hivecentral$
dhcp-128-83-55-98:Tet_Genes_Nikita hivecentral$
dhcp-128-83-55-98:Tet_Genes_Nikita hivecentral$ blastn -query Tet_A.txt -db SS1_concat.fna
BLASTN 2.2.31+


Reference: Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb
Miller (2000), "A greedy algorithm for aligning DNA sequences", J
Comput Biol 2000; 7(1-2):203-14.
```

Appendix B: Details on tetracycline resistance genes

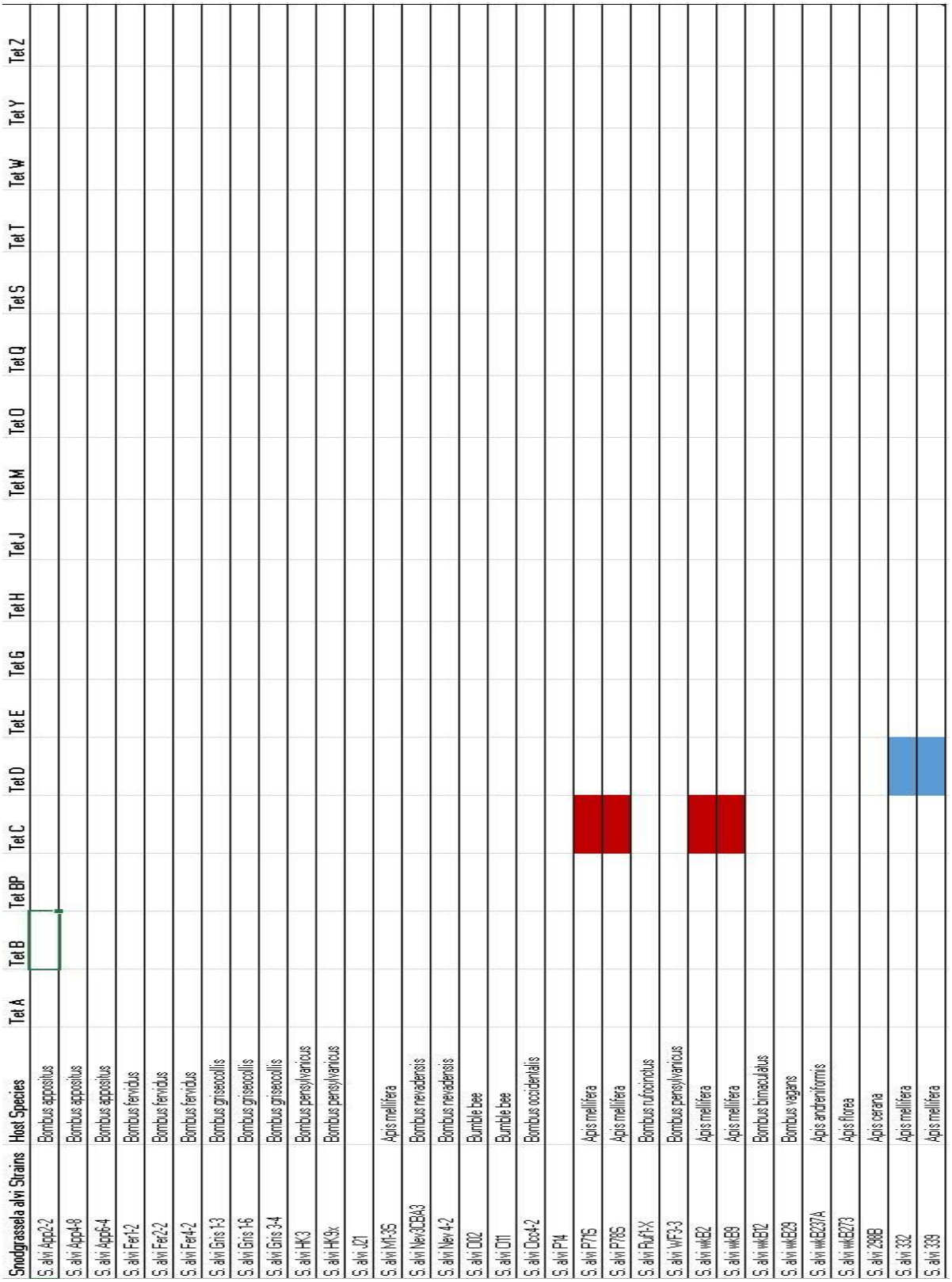
Gene of Interest	Species of Origin	Range (Length)	GenBank #
Tet A	*Pseudomonas aeruginosa*	1-1200 (1200)	X75761
Tet B	*Transposon Tn10*	1608-2813 (1206)	J01830
Tet C	*Escherichia coli (plasmid pBR322)*	86-1276 (1263)	J01749
Tet D	*Salmonella ordonez*	1521-2705 (1237)	X65876
Tet E	*Escherichia coli*	21-1238 (1218)	L06940
Tet G	*Vibrio anguillarum*	495-1676 (1183)	S52437
Tet H	*Pasteurella aerogenes*	986-2164 (1179)	AJ245947
Tet J	*Proteus mirabilis*	1084-2280 (1197)	AF038993
Tet Y	*IncQ plasmid pIE1120*	1680-2855 (1176)	AF070999
Tet Z	*Corynebacterium glutamicum*	1-1155 (1155)	AF121000
Tet M	*Enterococcus faecalis*	520-2439 (1920)	M85225
Tet O	*Streptococcus pneumoniae*	125-2044 (1920)	Y07780
Tet BP	*Clostridium perfringens*	1063-2325 (1263)	L20800
Tet Q	*Bacteroides fragilis*	314-2287 (1974)	Z21523
Tet S	*Listeria monocytogenes*	447-2372 (1926)	L09756
Tet T	*Streptococcus pyogenes*	478-2433 (1956)	L42544
Tet W	*Butyrivibria fibrisolvens*	3687-5606 (1920)	AJ222769
	Blue: Gram Neg Efflux protein		
	Green: Ribosomal Protiens		

Appendix C: Host and strain information for tested *G. apicola* genomes



Gilliamella apicola Stra	Host Species	Tet A	Tet B	Tet BP	Tet C	Tet D	Tet E	Tet G	Tet H	Tet J	Tet M	Tet O	Tet Q	Tet S	Tet T	Tet W	Tet Y	Tet Z
Orbales bimp	*Bumble Bee*																	
Orbus hercynius	*Bumble Bee*																	
G. apicola wkB308	*Apis cerana*																	
G. apicola wkB232	*Apis cerana*																	
G. apicola wkB233A																		
G. apicola wkB195	*Apis cerana*																	
G. apicola wkB178	*Apis dorsata*																	
G. apicola wkB171	*Apis andreniformis*																	
G. apicola wkB112	*Apis dorsata*																	
G. apicola wkB108	*Apis dorsata*																	
G. apicola wkB72	*Apis cerana*																	
G. apicola wkB30																		
G. apicola wkB18	*Bombus ruderatus*																	
G. apicola wkB11																		
G. apicola wkB7	*Apis mellifera*																	
G. apicola wkB1	*Apis mellifera*																	
G. apicola WF3-4	*Bombus pensylvanicus*																	
G. apicola P83G	*Apis mellifera*																	
G. apicola P62G	*Apis mellifera*																	
G. apicola P54G	*Apis mellifera*																	
G. apicola P46G	*Apis mellifera*																	
G. apicola P17																		
G. apicola Occ4-3	*Bombus occidentalis*																	
G. apicola Occ3-1	*Bombus occidentalis*																	
G. apicola Nev 6-6	*Bombus nevadensis*																	
G. apicola Nev 5-1	*Bombus nevadensis*																	
G. apicola Nev 3-1	*Bombus nevadensis*																	
G. apicola M6-3G	*Apis mellifera*																	
G. apicola M6IG																		
G. apicola M1-2G	*Apis mellifera*																	
G. apicola Impl-6	*Bombus impatiens*																	
G. apicola Impl-1	*Bombus impatiens*																	
G. apicola I20	*Bumble Bee*																	
G. apicola HK7	*Bombus pensylvanicus*																	
G. apicola HK2	*Bombus pensylvanicus*																	
G. apicola Gris 3-2	*Bombus griseocollis*																	
G. apicola Gris 1-4	*Bombus griseocollis*																	
G. apicola GillEmp13	*Bombus impatiens*																	
G. apicola Fer 4-1	*Bombus fervidus*																	
G. apicola Fer2-1	*Bombus fervidus*																	
G. apicola Fer1-1	*Bombus fervidus*																	
G. apicola Choc 6-1	*Bombus impatiens*																	
G. apicola Choc 5-1	*Bombus impatiens*																	
G. apicola Choc4-2	*Bombus impatiens*																	
G. apicola Choc3-5	*Bombus impatiens*																	
G. apicola Bim3-2	*Bombus bimaculatus*																	
G. apicola Bim1-2	*Bombus bimaculatus*																	
G. apicola Bif1-4	*Bombus bifarius*																	
G. apicola B02																		
G. apicola App6-5	*Bombus appositus*																	
G. apicola App4-10	*Bombus appositus*																	
G. apicola App2-1	*Bombus appositus*																	
Frischella perrara PEB0191	*Bumble Bee*																	

Appendix D: Host and strain information for tested *S. alvi* genomes



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Biography:

Nikita B. Prasad was born in San Antonio, Texas on October 21, 1994 and moved to Austin in 1995. She was a student at the Kealing Magnet Middle School and Liberal Arts and Science Academy High School in Austin before pursuing her undergraduate studies. She enrolled in the Plan II Honors program and Dean's Scholars Natural Science Honors program at the University of Texas at Austin in 2013 and studied biology. With ambitions to go to medical school, she was a member of Plan II premed, and Dr. Moran and Dr. Palmer's microbiology labs to study the effects of antibiotic resistance in microbial communities. She also maintained her interests in the fine arts by becoming an Indian classical dance teacher at the Natyalaya School of Dance throughout her college years. She loves working with kids and upon graduating with Honors Biology, Nikita plans to pursue a medical degree in pediatrics at the University of Texas Medical Branch medical school in the fall.